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Regulation of cytosolic calcium in skeletal muscle cells of the *mdx* mouse under conditions of stress

Wim J. Leijendekker, Anne-Catherine Passaquin, [2]Laurent Metzinger & [1]Urs T. Rüegg

Pharmacology Group, School of Pharmacy, University of Lausanne, 1015 Lausanne, Switzerland

1 In Duchenne muscular dystrophy (DMD) dysregulation of cytosolic calcium appears to be involved in the degeneration of skeletal muscle fibres. Therefore, we have studied the regulation of the free cytosolic calcium concentration ($[Ca^{2+}]_c$) under specific stress conditions in cultured myotubes isolated from the hind limbs of wild-type (C57BL10) and dystrophin-deficient mutant *mdx* mice. $[Ca^{2+}]_c$ in the myotubes was estimated by the use of the Ca^{2+}-sensitive fluorescent dye, fura-2.

2 Resting $[Ca^{2+}]_c$ was similar in *mdx* and normal myotubes (35 ± 9 nM and 38 ± 11 nM, respectively). However, when *mdx* myotubes were exposed to a high extracellular calcium concentration ($[Ca^{2+}]_e$) of 40 mM, the $[Ca^{2+}]_c$ was elevated to 84 ± 29 nM, compared to 49 ± 7 nM in normal myotubes.

3 Lowering the osmolarity of the superfusion solution from 300 mOsm to 100 mOsm resulted also in a rise in $[Ca^{2+}]_c$ which was about two times higher for *mdx* (243 ± 65 nM) than for C57BL10 (135 ± 37 nM). Replacing extracellular Ca^{2+} by EGTA (0.2 mM) prevented the rise in $[Ca^{2+}]_c$ in both *mdx* and normal myotubes when exposed to the low osmolarity solution.

4 Gadolinium ion (50 μM), an inhibitor of Ca^{2+} entry, antagonized the rise in $[Ca^{2+}]_c$ of myotubes superfused with 40 mM $[Ca^{2+}]_e$ by 20-40% for both *mdx* and C57BL10 cells, but did not significantly reduce the rise in $[Ca^{2+}]_c$ when the cells were exposed to the hypo-osmotic buffer (100 mOsm).

5 Incubation of the cell culture for 3-5 days from the onset of induction of myotube formation with the membrane permeable protease inhibitor, calpeptin (50 μM) abolished the rise in $[Ca^{2+}]_c$ in *mdx* myotubes upon exposure to hypo-osmotic shock.

6 Treatment of the cell culture for 3-5 days with α-methylprednisolone (PDN, 10 μM) attenuated the rise in $[Ca^{2+}]_c$ following hypo-osmotic stress for both normal and *mdx* myotubes by about 50%.

7 The results described here suggest an increased permeability of *mdx* myotubes to Ca^{2+} under specific stress conditions. The ameliorating effect of PDN on $[Ca^{2+}]_c$ could explain, at least partly, the beneficial effect of this drug on DMD patients.

Keywords: Duchenne muscular dystrophy; dystrophin deficiency; *mdx* mouse; skeletal muscle; cytosolic calcium; hypo-osmotic stress; prednisolone; calpeptin; gadolinium

Introduction

Duchenne muscular dystrophy (DMD) patients and *mdx* mice suffer from a genetic disorder characterized by the absence of dystrophin, a cytoskeletal protein (Hoffman *et al.*, 1987; Koenig *et al.*, 1987). Dystrophin is associated by its C-terminal domain with a complex of sarcolemmal glycoproteins, i.e. dystrophin associated glycoproteins (DAGs) which in turn bind the extracellular matrix protein merosin (muscle type of laminin) (Campbell & Kahl, 1989). Since the N-terminal portion of dystrophin has been proposed to interact with cytoskeletal actin (Hemmings *et al.*, 1992), the dystrophin-glycoprotein complex acts as a transsarcolemmal linker between the subsarcolemmal cytoskeleton and the extracellular matrix (Ervasti & Campbell, 1991; 1993). Therefore it has been hypothesized that dystrophin supports the mechanical stability of the muscle membrane (Menke & Jockusch, 1991). Also, a recent study (Yang *et al.*, 1995) indicates that dystroglycan, a component of the DAG complex, could be implicated in signal transduction due to a direct interaction with Grb2, an adapter protein of the ras pathway.

The precise mechanism, however, by which the lack of dystrophin causes muscle degeneration is unknown. One idea is that under mechanical stress the membrane of dystrophin-deficient muscle is more fragile than that of normal muscle, resulting in nonspecific plasma membrane lesions (Duncan, 1989; Petrof *et al.*, 1993). Another view argues that the absence of dystrophin is associated with changes in the function of specific membrane proteins, leading to an impaired calcium regulation (Fong *et al.*, 1990; Franco, Jr. & Lansman, 1990). Free cytosolic calcium levels ($[Ca^{2+}]_c$) were found to be elevated within muscle cells of DMD patients and of *mdx* mice compared to controls (Turner *et al.*, 1988; Bakker *et al.*, 1993). This phenomenon seems to be specific to Ca^{2+} since the permeability to sodium and the intracellular sodium concentration were the same for dystrophin-deficient and control mice (Turner *et al.*, 1991). Moreover, an increased activity of calcium leak channels (Fong *et al.*, 1990), and of calcium-sensitive stretch-inactivated ion channels has been reported (Franco, Jr. & Lansman, 1990). Transgenic introduction of dystrophin restores $[Ca^{2+}]_c$ and calcium leak channel activities of *mdx* cells to normal levels (Denetclaw, Jr. *et al.*, 1994), underlining the effect of dystrophin on $[Ca^{2+}]_c$ regulation. The elevated $[Ca^{2+}]_c$ in DMD or *mdx* muscle fibres is thought to initiate Ca^{2+}-activated proteolysis, which is assumed to play a major role in the process of cell degradation (Duncan, 1978; Turner *et al.*, 1988; MacLennan *et al.*, 1991). However, several authors were unable to show an enhanced $[Ca^{2+}]_c$ in the dystrophin-deficient state (Rivet-Bastide *et al.*, 1993; Gailly *et al.*, 1993; Head, 1993; Pressmar *et al.*, 1994) and consequently the role of calcium in the pathophysiological process and the significance of an altered cell membrane permeability in the degenerative process in DMD remains to be clarified.

In the present study, $[Ca^{2+}]_c$ homeostasis was examined in single myotubes in primary cultures isolated from *mdx* and C57BL10 newborn mice. To assess the effect of stress on Ca^{2+} regulation in these myotubes, two different conditions were

[1] Author for correspondence.
[2] Present address: Molecular Genetics Group, Institute of Molecular Medicine, John Radcliffe Hospital, Oxford OX3 9DU.

examined. Cells were either exposed to an increased level of extracellular Ca^{2+} ($[Ca^{2+}]_e$) or were challenged by exposure to a solution of reduced osmolarity (Menke & Jockusch, 1991), which resulted in cell swelling. Subsequently, the role of Ca^{2+} influx into the myotubes was studied by exposing cells either to EGTA or to Gd^{3+}, an inhibitor of stretch-sensitive channels (Yang & Sachs, 1989; Franco-Obregon Jr & Lansman, 1994). Finally, we have tested the effect of two treatments which could positively affect $[Ca^{2+}]_c$ handling of *mdx* myotubes, i.e. glucocorticoid treatment and the inhibition of Ca^{2+}-activated proteolysis. Glucocorticoid therapy has been reported to have a beneficial effect on DMD patients (Mendell *et al.*, 1989; for review: Kahn, 1993). Moreover, we recently have shown that in the C2 mouse skeletal muscle cell line, the glucocorticoid, α-methylprednisolone (PDN), led to a 2 fold decrease in $[Ca^{2+}]_c$ when these cells were treated for at least 3 days (Metzinger *et al.*, 1995). Since an increased proteolysis could be involved in $[Ca^{2+}]_c$ dysregulation in *mdx* myotubes (Turner *et al.*, 1988; 1993), we have also investigated the effect of calpeptin, an inhibitor of Ca^{2+}-activated proteases (Mehdi, 1991; Spencer *et al.*, 1995).

Methods

Cell cultures

Primary cultures of mouse skeletal muscle cells were prepared from hind leg muscles of 1–5 day-old normal (wild-type, C57BL10/C57BL10) and *mdx* (C57BL10/*mdx*) mice as described (Passaquin *et al.*, 1993). Following trypsinization of the muscles, cells were plated in medium 199 (Gibco, Basel, Switzerland) supplemented with 10% horse serum (Gibco) and 10 mg l^{-1} ciproxine (Bayer, Zürich, Switzerland), and incubated at 37°C in a water-saturated atmosphere of 95% air and 5% CO_2. Cells were grown on glass coverslips (18 × 18 mm) coated with 2% gelatin (Sigma, Buchs, Switzerland). Fusion of myoblasts into myotubes started at day 3 in culture.

Culture treatment

α-Methylprednisolone (PDN) was obtained from Upjohn Pharma (Brüttisellen, Switzerland) and calpeptin was purchased from Sigma (Buchs, Switzerland). PDN was added at the beginning of myoblast fusion (i.e. 3rd day of culture) at a final concentration of 10 μM. Calpeptin (50 μM final concentration) was added at day 4 of culture and cells were used for the experiments between day 7 and 10 in culture.

Measurements of the cytosolic calcium concentration

$[Ca^{2+}]_c$ measurements were performed on single myotubes loaded with the fluorescent dye fura-2. Cells attached to a glass coverslip (18 × 18 mm) were washed 2 times with control physiological salt solution (PSS) buffer (Table 1). The osmolarity was determined by the technique of lowering the freezing-point (osmometer; Infochroma, Zug, Switzerland). Cells were incubated for 45 min with fura-2/AM (10 μM) (Molecular Probes Inc., Eugene, OR, U.S.A.) in control PSS buffer, in the dark and at room temperature. The coverslip was then transferred to a superfusion chamber in which the cells were superfused with control PSS buffer. The chamber was mounted on the stage of a fluorescent microscope (Nikon Diaphot, Küsnacht, Switzerland) and the slit was adjusted so as to illuminate one single myotube. All measurements were performed on single rod-shaped myotubes of similar diameter (about 20 μm, whereas the length varied between 100 to 300 μm). The fluorescence was induced by exciting the myotube at alternating wavelengths of 340 and 380 nm and the measurements of the emitted light were made at 510 nm. The ratio R of the emitted light (510 nm) at 340 and 380 nm was determined using the PhoCal software (Life Science Resources Ltd., Cambridge, UK) and was used to calculate $[Ca^{2+}]_c$ with the following equation: $[Ca^{2+}]_c = K_d\beta(R - R_{min})/(R_{max} - R)$ (Grynkiewicz *et al.*, 1985), where R_{max} is the fluorescence ratio under saturating $[Ca^{2+}]_c$, R_{min} the same ratio under Ca^{2+}-free conditions, β the ratio of the fluorescence at 380 nm at maximum and minimum $[Ca^{2+}]_c$ levels, and K_d the apparent equilibrium constant (taken to be 224 nM) (Grynkiewicz *et al.*, 1985). Calibration was performed by superfusing the cells with PSS buffer containing ionomycin (20 μM) to obtain R_{max} and by superfusion with 5 mM EGTA (20–30 min) to estimate R_{min}. Finally the background fluorescence signal was determined by quenching the fluorescence with 2 mM $MnCl_2$ in PSS buffer without $CaCl_2$. The background fluorescence was subtracted from all previous measurements.

Experimental protocols

In one set of experiments the myotubes were exposed to PSS buffer with various concentrations of external $CaCl_2$. $CaCl_2$ of the control PSS buffer (Table 1) was reduced to 0.12 mM or increased to 12 mM, without changing the other components of the buffer, or increased to 40 mM $CaCl_2$ while changing the NaCl concentration (buffer 1, Table 1). The following protocol was used: PSS buffer containing 0.12 mM $CaCl_2$ (5 min), control buffer (2 min), PSS buffer containing 12 mM $CaCl_2$ (2 min), buffer 1 (2 min), control buffer (5 min), calibration procedure (see above). The effect of reduced osmolarity was examined by exposing myotubes to low osmolarity buffers (buffer 2 or buffer 3; Table 1) followed by exposure to buffer with 40 mM $CaCl_2$ (buffer 1). The following experimental protocol was used: control buffer (2 min), buffer 2 (5 min), buffer 3 (10 min), control buffer (5 min), buffer 1 (3 min), control buffer (5–10 min), calibration procedure.

Statistics

All values are expressed as means ± s.d. and Student's *t* test was used for statistical analysis.

Results

Resting cytosolic calcium concentration

The fluorescence intensity ratio ($R_{340/380}$) at rest was found to be slightly higher in *mdx* (0.51 ± 0.07; $n = 12$) than in C57BL10 myotubes (0.45 ± 0.03; $n = 10$), although this was not significantly different. Likewise the R_{min} values appeared to some extent to be elevated in *mdx* myotubes (0.39 ± 0.07, $n = 10$) compared to C57BL10 (0.33 ± 0.03, $n = 10$). $[Ca^{2+}]_c$ from *mdx* and C57BL10 myotubes were calculated using the calibration parameters of R_{min} and R_{max} (4.9 ± 1.1 and 6.5 ± 2.6, for *mdx* and C57BL10, respectively) and β (6.2 ± 1.7 and 7.5 ± 2.4, for *mdx* and C57BL10, respectively) determined in permeabilized cells. Hence, using these parameters for calculation of $[Ca^{2+}]_c$, similar values of basal $[Ca^{2+}]_c$ were found for both *mdx* and C57BL10 myotubes (Figure 1).

Table 1 Composition of experimental buffers: all were at pH 7.4

	Control buffer mM	*Buffer 1* mM	*Buffer 2* mM	*Buffer 3* mM
HEPES	5	5	5	5
KCl	5	5	5	5
$MgCl_2$	1	1	1	1
NaCl	145	85	6	6
$CaCl_2$	1.2	40	1.2	1.2
Glucose	10	10	1	1
Sucrose	–	–	100	50
Osmolarity	300 mOsm	308 mOsm	155 mOsm	100 mOsm

Effects of stress on $[Ca^{2+}]_c$

An increase in extracellular calcium concentration ($[Ca^{2+}]_e$) from 0.12 mM to 1.2 mM or from 1.2 mM to 12 mM resulted in a slight rise in $R_{340/380}$ and $[Ca^{2+}]_c$ in myotubes from both *mdx* and C57BL10 mice (Figure 1). A further augmentation of $[Ca^{2+}]_e$ to 40 mM resulted in a significant increase in $R_{340/380}$ in *mdx* myotubes compared with values at rest (from 0.51 ± 0.07 to 0.66 ± 0.13, $P < 0.01$) whereas no increase in $R_{340/380}$ of normal cells was detected (0.45 ± 0.03 to 0.48 ± 0.03). Consequently, $[Ca^{2+}]_c$ was significantly elevated (+70%) in dystrophin-deficient myotubes.

Exposure of myotubes to hypo-osmotic solutions (155 mOsm and 100 mOsm) resulted in a transient rise in $[Ca^{2+}]_c$, which was most striking in *mdx* myotubes. The maximum level of $[Ca^{2+}]_c$ reached during the time of exposure to a solution of 155 mOsm was not significantly different between *mdx* and C57BL10 myotubes, but at 100 mOsm $[Ca^{2+}]_c$ was twice as high in the *mdx* myotubes (101 ± 22 nM compared to 48 ± 8 nM for C57BL10, $P < 0.01$; Figure 2). When myotubes were superfused with the low Na^+ solution (6 mM Na^+) of normal osmolarity (300 mOsm by substitution with sucrose, 240 mM), the maximal change in $R_{340/380}$ as compared to control PSS buffer (145 mM NaCl) was less than 7% (amounting to a change in $[Ca^{2+}]_c$ of less than 10 nM) for both *mdx* ($n = 3$) and C57BL10. Following the hypo-osmotic shock (100 mOsm) cells were allowed to recover for about 5–10 min, which resulted in a further decrease in $[Ca^{2+}]_c$, although levels stayed above resting levels (by up to 60%). Additional exposure of the myotubes to high $[Ca^{2+}]_e$ (40 mM) resulted in an even more dramatic rise in $[Ca^{2+}]_c$ within the *mdx* myotubes (Figure 2).

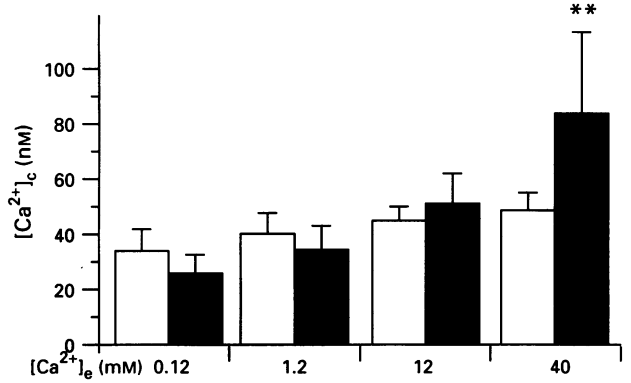

Figure 1 Effect of the extracellular Ca^{2+} concentration ($[Ca^{2+}]_e$) on $[Ca^{2+}]_c$ in C57BL10 (open columns, $n = 11$) and *mdx* myotubes (solid columns, $n = 10$). $[Ca^{2+}]_e$ was increased stepwise and the fluorescence ratio was determined during 2–3 min at each step (see Methods). **Value significantly different from normal ($P < 0.01$, Student's t test).

Effect of extracellular calcium

Superfusion of cells with PSS buffer containing EGTA (0.2 mM) and no $CaCl_2$ did not affect the $[Ca^{2+}]_c$ of either experimental group (Figure 3). Exposing cells to a buffer of 100 mOsm led to a doubling of $[Ca^{2+}]_c$ in *mdx* myotubes while no significant increase was noted in control cells. This increase in $[Ca^{2+}]_c$ in dystrophin-deficient cells following the hypo-osmotic shock was completely prevented when low osmotic PSS buffer containing EGTA was used (Figure 3).

Gd^{3+} (50 μM), a known inhibitor of stretch-sensitive leak channels (Yang & Sachs, 1989; Franco, Jr. *et al.*, 1991), inhibited the increase in $[Ca^{2+}]_c$ by about 50% when superfused with the 100 mOsm buffer in 6 of the 8 *mdx* cells tested, whereas in the other 2 cells $[Ca^{2+}]_c$ stayed high (about 120 nM) (Figure 4). Following a recovery period and subsequent exposure of the myotubes to a superfusion buffer containing 40 mM $[Ca^{2+}]_e$, $[Ca^{2+}]_c$ increased significantly less in both *mdx* and C57BL10 cells due to the presence of Gd^{3+}.

Myotubes cultured in the presence of calpeptin

Calpeptin (50 μM final concentration) was added to the cultures at the time of myoblast fusion, i.e. 3 or 4 days after cell seeding. Microscopic observations of the culture indicated that myotube formation was inhibited by about 50% for *mdx* and C57BL10 cell cultures (not shown). $[Ca^{2+}]_c$ measurements were performed on myotubes which appeared morphologically mature. Resting $[Ca^{2+}]_c$ in the myotubes treated with calpeptin during 3–5 days was significantly reduced in *mdx* cells (from 49 ± 18 nM ($n = 6$) to 24 ± 12 nM ($n = 6$)), but no decrease was noted in C57BL10 cells (not shown). The peak level of $[Ca^{2+}]_c$ following a hypo-osmotic shock was dramatically reduced in calpeptin-treated *mdx* myotubes, from 146 ± 68 nM ($n = 6$) to 48 ± 17 nM ($n = 6$) for untreated and treated myotubes respectively (Figure 5).

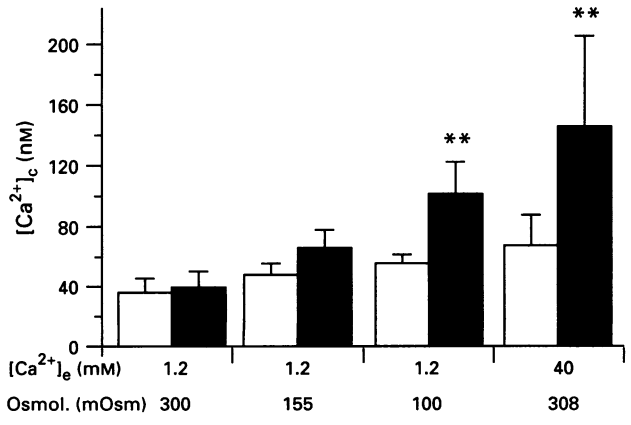

Figure 2 Effect of reduced osmolarity on $[Ca^{2+}]_c$ in C57BL10 (open columns) and *mdx* (solid columns) myotubes. In order to examine changes in $[Ca^{2+}]_c$ due to osmotic stress on the membrane, cells were exposed to two grades of hypo-osmotic shock (155 mOsm (5 min) and 100 mOsm (10 min), respectively), allowed to recover for 5–10 min by superfusion with control buffer (5–10 min), and then exposed (3 min) to a 40 mM $[Ca^{2+}]_e$ solution (see Methods). **Values significantly different ($P < 0.01$), *mdx* ($n = 12$) compared to C57BL10 ($n = 8$).

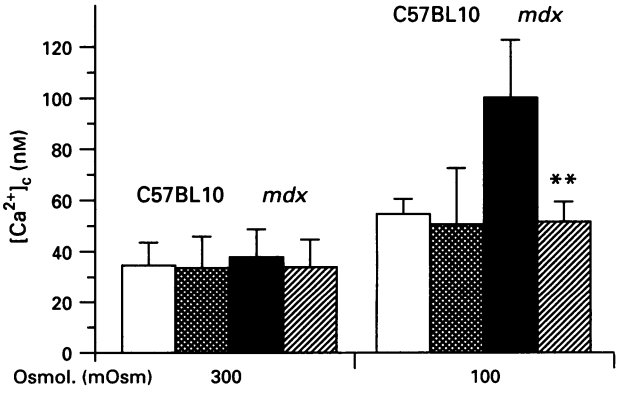

Figure 3 Effect of extracellular Ca^{2+} on $[Ca^{2+}]_c$ changes due to a hypo-osmotic shock. The myotubes were superfused for a period of 3 min with control PSS buffer (300 mOsm) containing either 1.2 mM $[Ca^{2+}]_e$ or 0.2 mM EGTA without $CaCl_2$ and then during 10 min with the low osmotic buffer containing 1.2 mM $[Ca^{2+}]_e$ (100 mOsm) or 0.2 mM EGTA without $CaCl_2$ (100 mOsm). Open columns, C57BL10 + 1.2 mM Ca^{2+}; cross-hatched columns, C57BL10 + EGTA; solid columns, *mdx* + 1.2 mM Ca^{2+}; hatched columns, *mdx* + EGTA. **Value significantly different compared to *mdx* untreated ($P < 0.01$, $n = 4$ or higher).

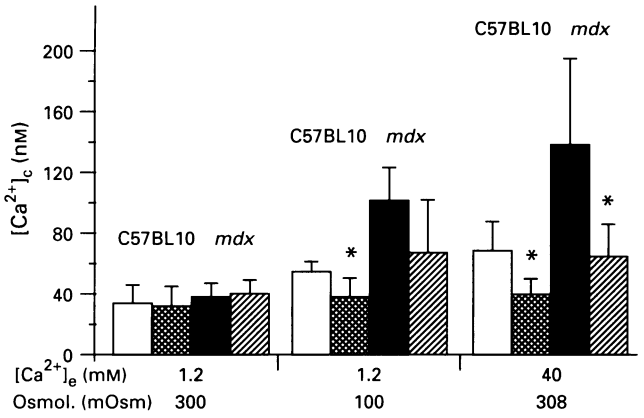

Figure 4 Effect of Gd^{3+} on resting $[Ca^{2+}]_c$ and on $[Ca^{2+}]_c$ upon exposure to a low osmotic buffer (100 mOsm) and to high $[Ca^{2+}]_e$. Osmotic stress and high $[Ca^{2+}]_e$ treatment was performed as described in the legend for Figure 2. Open columns, C57BL10; cross-hatched columns C57BL10 + Gd^{3+} (50 μM); solid columns, *mdx*; hatched columns, *mdx* + Gd^{3+} (50 μM). *Values significantly different compared to untreated myotubes ($P<0.05$, $n=5$ or higher).

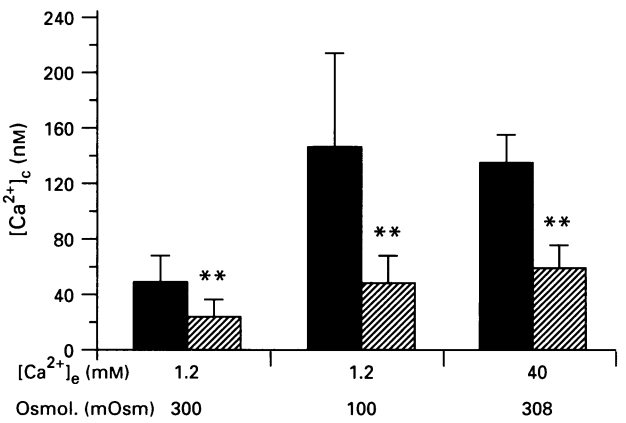

Figure 5 Effect of calpeptin treatment on basal $[Ca^{2+}]_c$ and $[Ca^{2+}]_c$ upon exposure of myotubes to a low osmotic solution. Myotubes were treated with calpeptin (final concentration of 50 μM) from day 4 on. Experiments were performed at days 7 to 10 by exposing cells to a 100 mOsm buffer and subsequently to the high $[Ca^{2+}]_e$ (40 mM) buffer. Osmotic stress and high $[Ca^{2+}]_e$ treatment was performed as described in the legend for Figure 2. Solid columns, *mdx*; hatched columns, *mdx* + calpeptin (50 μM). **Values significantly different compared to *mdx* untreated ($P<0.01$, $n=6$).

Myotubes cultured in the presence of PDN

Following the onset of myotube formation (day 3 or 4 after seeding), the cultures were incubated for 3 or 4 days with PDN (10^{-5} M). PDN significantly reduced basal $[Ca^{2+}]_c$ by 50% in C57BL10 myotubes (from 35±9 nM to 16±4 nM, $P<0.01$) but not in *mdx* cells (Figure 6). Moreover, upon changing to the low osmotic solution, $[Ca^{2+}]_c$ stayed considerably lower in myotubes treated with PDN for both *mdx* (−50%) and C57BL10 (−48%) cells than in untreated cells (Figure 6). However, peak $[Ca^{2+}]_c$ reached during the superfusion with the 100 mOsm buffer was still 45% higher in PDN-treated *mdx* cells when compared to PDN-treated C57BL10 cells. Also, following additional exposure of the cells to 40 mM $[Ca^{2+}]_e$, $[Ca^{2+}]_c$ stayed markedly lower for *mdx* myotubes treated with PDN (−58%) compared with untreated *mdx* cells (Figure 6).

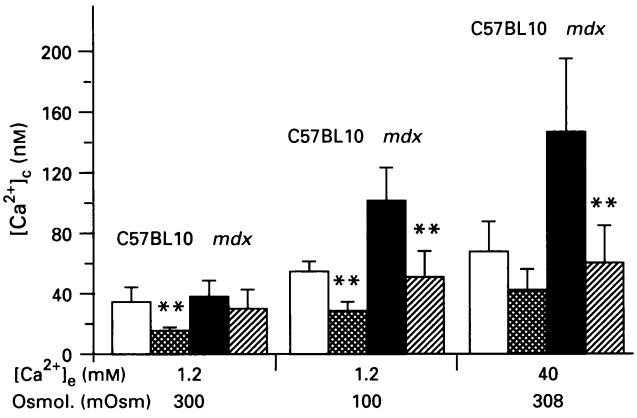

Figure 6 Effect of α-methylprednisolone (10^{-5} M) treatment of *mdx* and C57BL10 myotubes on $[Ca^{2+}]_c$ following exposure to a hypo-osmotic shock. PDN was added at day 3 or 4 of culture and measurements were performed between day 7 and 10 of culture. Open columns, C57BL10; cross-hatched columns, C57BL10 + PDN; solid columns, *mdx*; hatched columns, *mdx* + PDN. Osmotic stress and high $[Ca^{2+}]_e$ treatment was performed as described in the legend of Figure 2. *Values significantly different compared to untreated myotubes ($P<0.01$, $n=6$ or higher).

Discussion

The dysregulation of calcium homeostasis in dystrophin-deficient skeletal muscle cells derived from DMD patients and *mdx* mice is still an unresolved issue (Turner *et al.*, 1993; McArdle *et al.*, 1994; Franco-Obregon Jr & Lansman, 1994). While some authors have noted an increased cytosolic Ca^{2+} concentration in DMD and *mdx* myotubes at rest (Turner *et al.*, 1988; 1991; Bakker *et al.*, 1993), others have not been able to reproduce this (Gailly *et al.*, 1993; Head, 1993; Pressmar *et al.*, 1994). Our results indicate that resting $[Ca^{2+}]_c$ was not significantly altered in dystrophin-deficient murine myotubes. This discrepancy for basal $[Ca^{2+}]_c$ could be due to an effect of the dystrophin-deficient state on the calibration parameter R_{min} used for $[Ca^{2+}]_c$ calculation. Gailly *et al.* (1993) reported that $R_{340/380}$ was significantly enhanced in adult *mdx* skeletal muscle fibres as compared to controls, but since the R_{min} value determined within the fibres was also higher, no difference was found in the calculated basal $[Ca^{2+}]_c$. We have also observed higher $R_{340/380}$ and R_{min} values for *mdx* myotubes at rest as compared to C57BL10 myotubes. If this was taken into account for the calculation of $[Ca^{2+}]_c$ (see Methods), basal $[Ca^{2+}]_c$ was found to be the same for *mdx* and C57BL10.

When myotubes from *mdx* mice were subjected to two types of stress conditions, i.e. by exposing them to high $[Ca^{2+}]_e$ or to a hypo-osmotic shock, an impaired calcium handling was noted. A high $[Ca^{2+}]_e$ (40 mM) resulted in a significant increase in $[Ca^{2+}]_c$ in *mdx* myotubes but not in C57BL10. This can be explained by either an increased Ca^{2+} influx, a reduced Ca^{2+} efflux and/or an impaired Ca^{2+} sequestering mechanism into the intracellular Ca^{2+} pools under this condition. This effect of $[Ca^{2+}]_e$ suggests a dysregulation of $[Ca^{2+}]_c$ in *mdx* myotubes unrelated to mechanical function.

Imposing mechanical stress on the myotubes by swelling due to a hypo-osmotic solution resulted in a rise in $[Ca^{2+}]_c$ which was more dramatic in the *mdx* cells. Exclusion of calcium from the hypo-osmotic solution totally prevented the change in $[Ca^{2+}]_c$, indicating that Ca^{2+} release from internal Ca^{2+} stores was unlikely to be involved in the rise of $[Ca^{2+}]_c$ (Figure 3). Moreover, part of this $[Ca^{2+}]_c$ influx was inhibited by Gd^{3+} (Figure 4), suggesting entry of Ca^{2+} through stretch-sensitive channels (Yang & Sachs, 1989; Franco, Jr. *et al.*, 1991). The more significant augmentation in $[Ca^{2+}]_c$ in *mdx* cells upon the hypo-osmotic shock could therefore be the result of either an increased Ca^{2+} influx and/or a reduced Ca^{2+} efflux. An enhanced Ca^{2+} influx in *mdx* myotubes could be due to membrane leakage resulting from mechanical instability

(Menke & Jockusch, 1991; Petrof *et al.*, 1993) or more specifically to an elevated Ca^{2+} leak channel activity (Fong *et al.*, 1990). On the other hand an impaired sarcolemmal Ca^{2+}-pump activity in *mdx* cells could explain a reduced Ca^{2+} efflux from the cytosol. It is noteworthy that, at least in erythrocytes, the sarcolemmal Ca^{2+}-ATPase activity is highly sensitive to proteolysis by calpain (Salamino *et al.*, 1994), reducing its activity. In our preparations the sarcolemmal Ca^{2+}-pump activity is unknown and requires further investigation.

Alternatively, an enhanced Ca^{2+} leakage into the cytosol due to a leaky membrane in dystrophin-deficient myotubes could induce activation of Ca^{2+}-dependent proteases and alter leak channel activity as a consequence. An increased proteolytic activity, which was inhibited by leupeptin, has been correlated with a defect in muscle $[Ca^{2+}]_c$ in *mdx* and DMD myotubes (Turner *et al.*, 1993) and muscle-specific calpains have indeed been shown to be activated in *mdx* (Spencer *et al.*, 1995). Incubation of our primary myotube cultures with the calpain inhibitor, calpeptin, lowered resting $[Ca^{2+}]_c$ and prevented to a large extent the rise in $[Ca^{2+}]_c$ during hypo-osmotic stress, suggesting that an enhanced Ca^{2+}-dependent proteolysis was involved in mediating the elevated Ca^{2+}-levels in these myotubes. Turner *et al.* (1993) proposed that dystrophin may protect calcium leak channels from proteolysis. On the other hand, membrane fusion in chick embryonic myoblasts was found to be inhibited by calpeptin, indicating a role of calpains in this process (Kwak *et al.*, 1993). We have also found a reduction (by 50%) of myotube formation by calpeptin. Therefore, other interpretations to explain the reduction of $[Ca^{2+}]_c$, that we have observed in the myotubes treated with calpeptin, cannot be excluded.

Lowering of $[Ca^{2+}]_c$ by incubation of primary cultures with the glucocorticoid PDN is in full agreement with our previous results with the C2 skeletal muscle cell line (Metzinger *et al.*, 1995). Several hypotheses can be put forward for the underlying mechanism(s) of these observations. A direct effect of PDN on myogenesis has been reported (Vilquin *et al.*, 1992; Metzinger *et al.*, 1993; Passaquin *et al.*, 1993), reflected by an acceleration in the rate of fusion of myoblasts. The expression of the Ca^{2+}-transport system, i.e. a Ca^{2+}-ATPase, could be altered. Moreover prevention of oxidative damage to the cell membrane by PDN cannot be excluded since lazaroids, which are structurally related to PDN and are known antioxidants, exert similar effects on skeletal myogenesis (Metzinger *et al.*, 1994; Vernier *et al.*, 1995). Finally, a membrane stabilizing effect of PDN has been reported (Massa *et al.*, 1975) which could also play a role in the observed decrease in $[Ca^{2+}]_c$ (this study and (Metzinger *et al.*, 1995) and Ca^{2+} fluxes across the sarcolemmal membrane of C2 myotubes (Metzinger *et al.*, 1995).

An inhibition of Ca^{2+} influx into the muscular fibres of DMD patients could be of therapeutic value. As the result with calpeptin suggests, one of several pharmacological approaches would be to inhibit Ca^{2+}-activated proteases (for a review see Wang & Yuen, 1994), although care should be taken to avoid possible interference with the cell fusion process. Our results with PDN indicate that at least part of the beneficial effect of glucocorticoids in the therapy of DMD could be related to a favourable effect of this type of drug on the handling of $[Ca^{2+}]_c$ in myotubes.

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